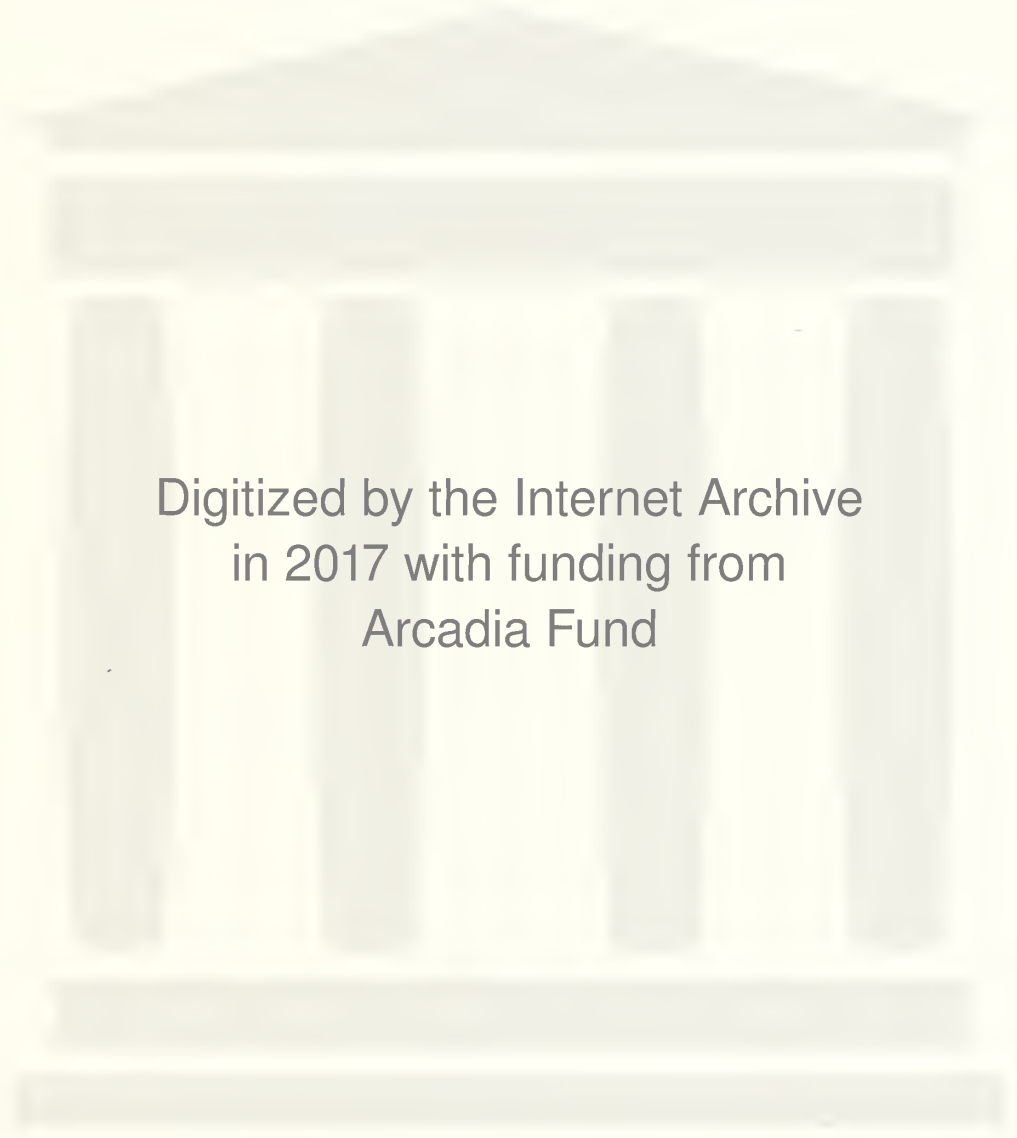

STUDIES ON THE RELATIONSHIP OF KETONE BODIES AND GROWTH HORMONE TO GLYCOGEN SYNTHESIS IN THE PERFUSED FROG'S HEART

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I. INTRODUCTION

A. History and Discussion of the Problem

1. The Question of the Conversion of Fatty Acids to Carbohydrate.

The ability of the animal body to produce a net synthesis of fat from ingested carbohydrate has been a well-established fact since the classical studies of Lawes and Gilbert (1, 2) over a century ago. These investigators demonstrated by balance experiments that animals deposited more fat than was fed in the diet. Further confirmation of the body's ability to transform dietary carbohydrates into fat was provided by Schoenheimer and Rittenberg's studies (3) in which deuterium was used as a tool to measure the synthetic process. The quantitative as well as the qualitative aspects of this conversion were further studied by Longenecker in 1939 (4).

However, it is much more difficult to demonstrate the reverse of this, the net synthesis by animal tissues of carbohydrate from fatty acids or from products of their metabolism. Though there have been several claims that such a synthesis does occur, the experimental evidence in general has been equivocal or open to much criticism. For every investigator who has claimed to have demonstrated this conversion it seems there has been someone else who has been unable to do so by using similar methods.

One of the earliest approaches to the problem of gluconeogenesis from fatty acids was the feeding of diets consisting solely of fats and then examining the liver for signs of increased

glycogen storage. By such methods Takao (5), Burn and Ling (6) and Magnusson (7) claimed large increases in liver glycogen. In contrast Bodey (8), Gregg (9) and Greisheimer (10) found little or no increase. The small increments in the latter experiments could readily be accounted for by the glycerol content of the fats, for the feeding of glycerol alone was found to produce a marked increase in liver glycogen (10). Even if the liver glycogen were consistently elevated in fat-fed animals it is not proof that the glycogen came from fats per se---it might have come from protein or from carbohydrates in other body tissues.

Other investigators have attempted to demonstrate by means of low respiratory quotients that fat can be converted to carbohydrate. By feeding high-fat diets Hawley, Johnson and Murlin (80) were able to produce respiratory quotients below 0.70 in humans and Hawley and Murlin (81) were able to do the same with a pig. Likewise Lyon, Dunlop and Stewart (82) found R. Q.'s as low as 0.59 in obese human subjects on reducing diets. These authors claim the low R. Q.'s obtained indicate a conversion of fat to carbohydrate. Their reasoning is as follows. Since the theoretical R. Q. for the oxidation of fat is 0.70 a non-protein R. Q. of less than 0.70 means the body is retaining excess O_2 to form oxygen-rich carbohydrate from fat.

However, is an R. Q. of less than 0.70 really proof of a gluconeogenesis from fat? Apparently not, for as Mitchell points out in his excellent review of the subject (11) gluconeogenesis from amino acids, desaturation of fatty acids and other intermediary oxidative reactions could also play a role in lowering

the R. Q. below 0.70. Also there are many errors in the correction of R. Q.'s for protein metabolism, one source of error being the body's delay in excreting urinary nitrogen. The nitrogen used in the calculation of R. Q.'s often comes in part from the catabolism of food given the animal in the meal prior to the test meal. Also, an R. Q. greater than 0.70 does not prove fat is not being converted to carbohydrate for a simultaneous oxidation of sugar would elevate the R. Q. Thus one cannot conclude definitely whether or not fats can give rise to carbohydrates by studying the respiratory quotients of animals.

Another approach has been that of liver perfusion studies. By such methods Burn and Marks (12) and Jost (13) claimed to be able to demonstrate the formation of carbohydrate in the liver from fat. However, Mitchell (11) criticizes these studies and claims that the glycogen increment may have been due to lactic acid formed while the experiments were being set up or from glycerol liberated from the fats present in the liver tissue. Gregg (14) in perfusion experiments with livers from fat-fed cats and dogs could not demonstrate any support for the theory of gluconeogenesis from fatty acids.

Some investigators have attempted to account quantitatively for the sugar excreted in the urine of depancreatized dogs in response to epinephrine injections. By such methods Chaikoff and Weber (18) concluded that in a few of their experiments the extra urinary sugar produced in response to the injection of epinephrine must have been derived in part from fatty acids. Yet in similar experiments Bollman, Mann and Wilhelmj (19)

could produce no evidence for the formation of sugar from fat.

In recent years many investigators have attacked the question of the conversion of fats to carbohydrates by means of studies with radioisotopes of carbon. In 1943 Buchanan et al. (20) fed carboxyl-labeled acetate, propionate and butyrate to fasted rats and found radioactivity in the liver glycogen. Here for the first time was conclusive evidence that the carbon atoms of short chain fatty acids can find their way into carbohydrate molecules. Strisower et al. (21) demonstrated in 1951 that C^{14} is found in the blood glucose of rats fed tripalmitin-6-C^{14}, a long chain fatty acid.

The mechanisms by which labeled carbon atoms of fatty acids find their way into carbohydrate molecules has been studied by numerous investigators (22 - 28) using isotopes of carbon. Their results indicate that the main pathway is the degradation of fatty acids into 2-carbon fragments (acetyl-CoA) which then traverse the Kreb's cycle and finally form glucose via the reverse of the Embden - Meyerhof scheme of glycolysis.

Now that it has been demonstrated that labeled carbons of fats find their way into carbohydrate molecules the question of net synthesis of sugar from fats arises. If it is assumed that for every two carbons entering the Kreb's cycle as acetyl-CoA two carbon atoms must be evolved as CO_2 obviously no net synthesis can take place. However, if substances other than acetate enter the cycle they could give rise to the liberated CO_2 while the carbons of acetate produced a net synthesis of carbohydrate. Whether or not such substances actually enter the Kreb's cycle

can be determined by measuring the CO_2 ratio (29) where

$$CO_2 \text{ ratio} = \frac{C^{14}O_2 \text{ derived from acetate-1-}C^{14}}{C^{14}O_2 \text{ derived from acetate-2-}C^{14}}.$$

If acetic acid is the only substance entering the Kreb's cycle the CO_2 ratio will equal 1.0 after approximately a dozen turns of the cycle. This can be demonstrated as follows: If one traces the carbon atoms of CH_3-*COOH (*designates a radioactive atom) through the citric acid cycle it will be seen that no labeled CO_2 is liberated after the first turn but that 100% of the C* is liberated as C^*O_2 after the second revolution. When C^*H_3-COOH is fed into the citric acid cycle no labeled CO_2 is set free during either the first or second turns. On the third turn 50% of the C* is liberated as C^*O_2 and after the 4th turn 75% has been given off. By the twelfth revolution 99.92% (29) of the labeled carbon has taken the form of carbon dioxide. With each subsequent turn the liberated C* approaches closer to 100% which is the value liberated when CH_3-C*OOH was fed into the cycle. Hence the CO_2 ratio approaches 1.0 after several turns of the cycle.

If unlabeled substances enter the Kreb's cycle at the same time as acetate-1-C^{14} or acetate-2-C^{14} they will dilute the specific activities of the Kreb's cycle intermediates. Weinman et al. (29) demonstrate mathematically that this would produce a CO_2 ratio greater than 1.0 when the inflow and outflow of labeled carbons has become constant.

Various investigators have made actual measurements of

the CO_2 ratio by using fasted rats (30), sheep lactating mammary gland slices (31), lactating cows (32) and rat liver slices (33, 34). The values obtained have ranged from 1.0 to 4.8. Hence it would appear that acetate is not the only source of carbon atoms entering the Kreb's cycle. Presumably substances such as glutamate and aspartate can enter the cycle by forming alpha-ketoglutarate and oxaloacetate respectively. Likewise proline, hydroxyproline, ornithine, arginine and histidine can enter the cycle by first forming glutamate as a metabolic breakdown product.

Also Weinman et al. (29) point out that from various experimental observations and thermodynamic considerations the reaction

$$\text{Oxaloacetate} + \text{ATP} \rightleftharpoons \text{Phosphopyruvate} + \text{ADP} + CO_2$$

proceeds more rapidly to the right than to the left, at least in the mammalian liver. Thus it would appear that fatty acids can be degraded to 2-carbon acetyl fragments which can then enter the Kreb's cycle to form oxaloacetate. For each of these acetyl fragments entering the cycle two carbons from substances such as glutamate or aspartate are liberated as CO_2. The oxaloacetate then gives rise to a net synthesis of carbohydrate. In summary, for every two carbons of fat forming glucose two carbons from another compound such as an amino acid must be utilized to maintain the Kreb's cycle. Hence it is possible to produce a synthesis of carbohydrate from fat via the Kreb's cycle though such a synthesis is not the same as the net gain of

fat from carbohydrate where a third substance (protein) is not needed.

2. Ketone Bodies and Their Relationship to the Glycogen Content of the Heart and Other Tissues.

Not only has there been much interest in the conversion of fatty acids to sugar but also in the possible conversion of ketone bodies --- acetoacetic acid, beta-hydroxybutyric acid and acetone --- to glucose since they are products of fat catabolism. Numerous investigators have demonstrated that acetoacetate arises largely by the recondensation of acetyl units formed in the degradation of fatty acids but that a small amount is formed from the beta-oxidation of butyric acid which is an incomplete breakdown product of fatty acids (22, 35, 36, 37, 38, 39). The utilization of acetoacetic acid in animal tissues is commonly ascribed to two types of metabolic processes --- reduction to beta-hydroxybutyric acid and oxidation to CO_2 and H_2O after degradation to acetyl fragments. The reduction and its reversibility was first observed by Friedmann and Maase (40) and Wakeman and Dakin (41). Its oxidative breakdown was demonstrated in experiments by Snapper and Grunbaum (42). To a small extent acetoacetic acid is decarboxylated to acetone by animal tissues but Sakami (43) has ruled out acetoacetate as the major source of acetone. The major precursor of acetone is not known. Some evidence has been presented that it may arise from leucine (44, 45).

Since acetoacetic acid or beta-hydroxybutyric acid is not

readily utilized by the liver (46) but is readily assimilated by striated muscle (47) and by cardiac muscle (48, 49) any conversion to glucose should be looked for in these extrahepatic tissues. Nagler (50) incubated surviving rat diaphragm in phosphate buffer medium containing glucose (200 mgm.%), glucose and insulin (0.05 unit per 1.0 ml), and glucose, insulin and acetoacetate (50 mgm.%). No effect of acetoacetate on glycogen synthesis was observed. Parnes and Wertheimer (51) and Chari and Wertheimer (53) reported that acetoacetate depressed glycogen synthesis in the rat diaphragm, even in the presence of insulin. Nor has anyone else been able to demonstrate a conversion of ketone to carbohydrate in striated muscle.

Of especial interest is the well extablished fact that conditions producing ketosis in animals cause an increase in the glycogen content of the myocardium. Long and Evans found in 1932 (52) that fasted rats have a higher cardiac glycogen level than fed animals. In 1944 Lackey et al. (54) found that rats made diabetic with alloxan likewise had a significantly elevated cardiac glycogen whereas the glycogen content of the liver and skeletal muscle was decreased in comparison to control animals.

In 1945 Lackey, Bunde and Harris (55) presented evidence that there is no correlation between cardiac glycogen and blood sugar or blood lactic acid levels but that there is a direct correlation between the blood ketone level and heart glycogen in rats under varied dietary conditions. There was no relationship between the concentration of blood ketone bodies and liver and muscle glycogen. Later this same group of investigators (56)

infused fasted and fed rats with sodium acetoacetate and sodium beta-hydroxybutyrate for four hours and found a marked increase in cardiac glycogen in each case. Of interest is the fact that in the fasted rats a much higher blood ketone level was needed to elevate the cardiac glycogen than in the case of the fed animals. Thus the blood ketone concentration does not appear to be the only factor controlling the level of cardiac glycogen in these experiments.

Since conditions producing ketosis unquestionably cause a rise in cardiac glycogen the possibility of a direct conversion of ketone bodies to glycogen in the heart naturally arises. Stadie and Haugaard in 1947 (57) could not demonstrate any glycogen synthesis in rat heart slices which were incubated with sodium beta-hydroxybutyrate. In 1956 Meyer and Bow (58) infused rats with Na acetoacetate-3-C^{14} and discovered no radioactivity whatsoever in the cardiac glycogen after 2.5 hours of infusion and concluded that "the net increase in heart carbohydrate accompanying ketonemia represents some secondary and yet undetermined influence of ketone bodies."

If there are any conditions under which the animal heart is able to convert ketone bodies to glycogen what pathways might be involved? Following are possibilities that have frequently been considered:

1) Acetoacetate → 2 acetyl-CoA → glucose via the Kreb's cycle and the reverse of the Embden - Meyerhof scheme of glycolysis.

2) Acetoacetate → oxaloacetate → glucose by omega oxidation of acetoacetate.

3) Acetoacetate → butyrate → succinate → oxaloacetate → glucose by omega oxidation of butyrate to succinate.

4) The Thunberg reaction, ie., the oxidative condensation of two moles of acetate to succinate (59).

5) (Acetoacetate →) acetone → pyruvate → glucose.

Meyer and Bow (60) perfused isolated dog's hearts with Na acetoacetate-3-C^{14} and found malate and succinate to be labeled almost exclusively in the carboxyl carbon which is compatible with pathway 1) and not 2) or 3). If 2) and 3) are mechanisms utilized by the heart acetoacetate-3-C^{14} should label malate and succinate in the methylene carbons. This was not the case. 3) is also unlikely in view of Lorber's demonstration (61) that butyrate is metabolized by animal tissues only in the conventional manner --- beta-oxidation to 2 acetyl-CoA. Meyer and Bow (60) claim to have demonstrated the same thing in the isolated dog's heart in an unpublished observation.

Though Foster has presented evidence that the Thunberg condensation does occur in the mold, Rhizopus nigricans, (62, 63) the reaction has never been demonstrated satisfactorily in animal tissues.

To a limited extent acetone gives rise to acetoacetate in the liver of rats by carboxylation (64). Also the acetone methyl carbon has been demonstrated to give rise to "formate", probably by a 2- and 1-carbon cleavage in which acetate is formed (43). But does acetone produce a 3-carbon intermediate of glycolysis such as pyruvate? If it does acetone labeled in the carbonyl position should label glucose according to the

following equation:

$$CH_3\text{-}C^{14}O\text{-}CH_3 \longrightarrow CH_3\text{-}C^{14}O\text{-}COOH \longrightarrow 2, 5 > 1, 6\text{-}C^{14} \text{ glucose.}$$

Metabolism of acetone by the carboxylation and cleavage mechanisms should label glucose solely in the 3, 4 positions. Accordingly Sakami and Lafaye (65) administered C^{14}-carbonyl-labeled acetone to rats and found higher activity in the 2, 5 than in the 1, 6 positions of glucose derived from liver glycogen. Thus there is evidence that to a certain extent animal tissues may be able to convert acetone to a 3-carbon intermediate of glycolysis. However, it has already been pointed out that acetone is not an important metabolic product of acetoacetate. Thus it would seem that for the greatest part any conversion of acetoacetate to glycogen in tissues under physiological conditions must follow the established pathway of beta-oxidation to 2 acetyl-CoA which then traverses the Kreb's cycle. As has already been pointed out, it is theoretically possible to produce an actual synthesis of glycogen by this pathway.

3. The Effect of Growth Hormone on Cardiac Glycogen.

If the heart does not synthesize glycogen from ketone bodies what is the explanation for the increased cardiac glycogen in animals with ketonemia? Certainly it is not due to increased insulin levels for insulin secretion is decreased in diabetes. Cruickshank and Startup concluded that the primary defect in the diabetic heart is a failure to oxidize carbohydrate, presumable secondary to a lack of insulin (66). Yet

is the increased cardiac glycogen in fasting animals due to insulin deficiency? Illingworth and Russell (67) gave fasting rats insulin and found no effect on the increased cardiac glycogen. Bogue et al. (68) concluded from studies on the dog heart-lung preparation that glucose and not lactate was a source of cardiac glycogen. Yet Lackey, Bunde and Harris (55) could find no correlation between cardiac glycogen levels and the concentration of glucose in the blood.

In 1951 Illingworth and Russell (67) found that fasted rats injected with growth hormone (1-2 mgm./100 Gm.) exhibited a marked increase in cardiac glycogen as compared to fasted controls. In 1956 Russell and Bloom (68) demonstrated that hypophysectomy entirely prevented the increment in cardiac glycogen normally observed in rats under fasting conditions, whereas the giving of growth hormone to hypophysectomized fasted rats restored the cardiac glycogen to the same levels seen in intact fasted controls. Adrenalectomy did not significantly affect the cardiac glycogen in either fasted or fed animals. Hence the presence of growth hormone appears essential to the production of the rise in heart glycogen observed in the fasting state. In unfasted animals hypophysectomy produced no change in cardiac glycogen or elevated it slightly. In rats previously fasted and then fed glucose growth hormone (1 mgm./100 Gm.) not only prevented the fall in cardiac glycogen normally observed under these conditions but caused it to rise above fasting levels. Adrouny and Russell (69) further demonstrated that growth hormone can

simulate the effects of fasting on the heart glycogen when given to fed animals and that feeding does not inhibit the activity of the hormone. In summary it appears that fasting may stimulate the secretion of growth hormone by the pituitary and it may be that diabetes and other conditions producing ketosis act likewise.

By what mechanism does growth hormone increase the glycogen content of the heart? Russell and Bloom suggest three possibilities:

1) Increased uptake of glucose from the blood.
2) Inhibition of utilization of stored glycogen.
3) Formation of glycogen from non-carbohydrate precursors within the heart.

In view of the anabolic action of growth hormone it is unlikely that it would produce gluconeogenesis from amino acids in the heart. Also there is no good evidence of the conversion of fats to glycogen in the heart under physiological conditions. Russell and Bloom point out that there is little evidence that growth hormone increases uptake of glucose by other tissues but that there is evidence that the hormone blocks carbohydrate utilization. Thus 2) would seem the most likely.

B. Purpose of the Present Investigation

Much evidence has been presented indicating that the heart is unable to synthesize glycogen from ketone bodies. Nevertheless, the question is not completely settled for it is not yet known whether a heart thoroughly depleted of its glycogen content and then given a ketone body as the sole metabolite can resynthesize glycogen. Admittedly this is subjecting the heart to rather unphysiological conditions. However, if carbohydrate is essential to normal cardiac function and if the heart has any enzyme system capable of producing gluconeogenesis from ketone bodies it should become evident under such demanding circumstances. Is there then a pathway, normally unused, by which the heart can produce glycogen in an emergency?

Accordingly, the present investigation was undertaken to determine if a heart so depleted of its glycogen can resynthesize this carbohydrate from a ketone body, beta-hydroxybutyric acid. The technique of heart perfusion seemed the best way to study this problem. The frog's heart was chosen because of the ease with which it can be perfused and because its metabolism in many ways is similar to that of the mammalian heart (70, 76, 77, 78, 79).

Since Bogue et al. (75) demonstrated quite conclusively that the heart is able to synthesize glycogen from glucose it seemed indicated to determine what effect, if any, ketone bodies have on this conversion. If ketone bodies are not converted to glycogen themselves do they increase the cardiac glycogen by means of a sparing action on glucose? In other words, can the

heart utilize ketone bodies for energy and thereby synthesize more glycogen from glucose than would be the case if glucose were present alone?

In view of the work by Russell et al. (67, 68, 69) demonstrating the glycogenic effect of growth hormone on heart muscle it was also decided to study the influence of this hormone on the possible synthesis of glycogen from ketones and from glucose in a frog's heart first depleted of all of its glycogen. Experiments to determine the effect of the hormone on cardiac glycogen in the intact frog were also performed.

II. MATERIALS AND METHODS

A. Animals --- American bullfrogs weighing 250 - 450 Gm. apiece were used in the experiments. Fresh shipments of frogs were obtained and used every two weeks. The animals were kept in a fasting state at a temperature of approximately 4° C.

B. Equipment:

1. Desiccator jar used as anaerobic chamber.
2. Perfusion apparatus as described below.

C. Chemicals used in depletion of cardiac glycogen and perfusion of the heart.

1. Nembutal (sodium pentobarbital).
2. Epinephrine -- 1:1000 concentration.
3. Glutathione -- reduced.
4. Beta-hydroxybutyric acid by Fisher Scientific Company.
5. Purified growth hormone made by Armour Laboratories.
6. Dextrose.
7. Physiological salt solution for perfusion as described by Clark et al. (70). Solution consisted of:

   NaCl, 0.65%

   $CaCl_2$, 0.024%

   KCl, 0.015%

   Na phosphate, 0.02% (pH = 7.6) in distilled H_2O.
8. Bottled oxygen and nitrogen gases.

D. Quantitative determination of tissue glycogen.

1. Chemicals:

   a) KOH, 3% and 30%

b) EtOH, 95%

c) HCl, 2.5%

d) Phenol red, 1%

e) Copper Reagent A

Na_2CO_3,	25.0 Gm./L
Rochelle salt	25.0 Gm./L
$NaHCO_3$	20.0 Gm./L
Na_2SO_4	200.0 Gm./L

f) Copper Reagent B

$CuSO_4 \cdot 5H_2O$) 15% with 1 - 2 gtts. conc. H_2SO_4/100ml.

g) Arseno Molybdate Soln.

Ammonium molybdate, 25 Gm. in 400 cc H_2O.

H_2SO_4, conc. 21 cc.

$Na_2HAsO_4 \cdot 7H_2O$, 3 Gm. in 25 cc H_2O.

Incubate at 37° for 24 - 48 hrs.

2. Methods:

The disintegration of tissue and hydrolysis of glycogen to glucose were carried out according to the method of Good, Kramer and Somogyi (71). Glucose was analyzed by the method of Nelson (74).

E. Perfusion Apparatus: The isolated beating heart was suspended in a container of physiological solution by means of cannulas inserted into the sinus venosus and into the two aortas. It was kept at a temperature of 25 - 27°C. by warm lights or by surrounding its container with cool water, depending on the room temperature. Situated above the heart was a flask containing the perfusate through which O_2 bubbled. From this

flask the solution dripped into the cannula inserted in the sinus venosus. The heart then pumped it against a resistance of about 1 cm. of H_2O into a second flask. From here it was blown by air under pressure back up to the first vessel. Thus the same solution could be recirculated through the heart as many times as was desirable.

Adequate oxygenation was assured by the bubbling of O_2 through the perfusate before it entered the heart and by blowing the fluid back up to the first flask by pressurized air. The resistance the heart pumped against was kept low to insure as nearly complete emptying of the ventricle with each contraction as was possible.

III. EXPERIMENTAL PROCEDURE

A. Depletion of Cardiac Glycogen.

The frogs were injected intraperitoneally with approximately 20 mgm. sodium pentobarbotal per 100 Gm. frog weight. After anesthesia occurred the heart was exposed by a ventral midline incision opening the peritoneal and pleural cavities. The abdominal and chest walls were reflected and the pericardium was stripped from the heart, the circulation of blood being left intact.

The frog with its heart thus exposed and still beating was placed ventral side up in a desiccator jar. The physiological salt solution described in the previous section was allowed to drip on the heart at a rate of 12 - 15 drops per minute from a container placed within the desiccator. Epinephrine was added to this solution at a concentration of 1/500,000 to stimulate the heart and a few mgm. of reduced glutathione were used to keep the epinephrine in a reduced state. The air in the desiccator was then displaced by 100% nitrogen and the chamber sealed to make it air tight. The heart, in situ in the frog and with isotonic solution dripping on it to prevent drying, continued to beat and pump blood in this anaerobic atmosphere.

After 30 minutes to 4 hours, sometimes longer, in this nitrogen-filled chamber a small area developed at the base of the ventricle which appeared white in contrast to the red color of the remaining myocardium. If the preparation was then removed to an aerobic atmosphere the white area of muscle disappeared

but reappeared shortly when the heart was reintroduced into the anaerobic chamber. As the heart continued to beat anaerobically the white area, which resembled scar tissue and did not contract, enlarged continuously until it involved the entire ventricle. At this stage the ventricle could not be revived by the introduction of oxygen.

When the white area which grossly resembled an old infarct reached a size about 3 X 3 mm. in area the entire ventricular myocardium could be shown to be depleted of glycogen (see under "Results"). The heart, still contracting actively, could then be removed from the anaerobic chamber and perfused as a preparation with ventricles free of glycogen. All glycogen determinations were carried out on viable ventricular myocardium not involved by the "infarct" (see "Discussion" for quotes). The auricles were not analyzed because of the difficulty in excluding sinus venosus and aortic tissue from the specimen. Also it would be difficult to be certain the ratio of ventricular to auricular tissue was the same each time. Hence, though the entire heart was perfused, only the ventricles were analyzed for glycogen. Only hearts which developed the "infarct" were perfused though it was found that some hearts became depleted of glycogen before they developed the white area. Other hearts never developed the scarred appearance until long after they had ceased beating from exhaustion.

B. Perfusion of the Heart.

Following the development of the "infarct" about 3 X 3 mm. in

area at the base of the ventricle the frog was removed from the anaerobic chamber. Immediately a glass cannula was inserted into the sinus venosus and the blood washed from the heart with the physiological solution. The two aortas were then cannulated with polyethylene tubing and the heart removed from the frog and attached to the perfusion apparatus. The heart was then perfused with 400 cc. of the physiological salt solution to which had been added substances such as glucose, beta-hydroxybutyric acid, etc. in known amounts. All solutions were adjusted to a pH of 7.6.

After the heart had been perfused for the desired length of time the ventricle, excluding the "infarcted" portion, was removed, blotted three times to remove excess fluid and dropped into a weighed centrifuge tube containing 2cc. of 30% KOH. The tube was reweighed rapidly and then heated over an open flame to disintegrate the tissue. Glycogen was then determined quantitatively. About 20 seconds elapsed from the time the ventricle was cut from the auricles to the time it was disintegrated by boiling.

In addition to the perfusions a set of in-vivo experiments was performed in which fasting frogs were injected intraperitoneally with either growth hormone or saline. The ventricles were then analyzed for glycogen in the two groups of animals and the results compared.

IV. RESULTS

The results of this investigation are summarized in Tables I to IV.

Table I:

It should be noted that two types of controls were used for the perfusion experiments. The values listed in line 1 were obtained on hearts removed from frogs under Nembutal anesthesia with immediate determination of ventricular glycogen. Thus the value of 1238 ± 74 mg% represents the glycogen content of the ventricles of frogs kept without food two weeks or less. Though not indicated on the table there was no correlation between the level of cardiac glycogen and the duration of fasting within this two week period. However, one frog sacrificed after three months of fasting (not included in Table I) had a ventricular glycogen content of 2450 mg%, more than 900 mg% greater than that of any frog in the same shipment kept less than two weeks. Unfortunately, no other animals were kept for such an extended period of time.

Line 2 of Table I demonstrates that hearts which were allowed to beat in an anaerobic atmosphere until the "infarct" developed and were then cannulated uniformly contained no glycogen in the ventricles. These hearts therefore serve as the controls for hearts that were treated in the same manner but were then perfused with various metabolites (lines 3 - 6 of Table I and Tables II and III). It is obvious that the 15 to 20 minutes in an aerobic

TABLE I

Determination of Glycogen Synthesis in Glycogen-Free Frog Hearts Perfused with Glucose and Na Beta-hydroxybutyrate

Experiment	Number of Animals	Ventricle Glycogen Mg%	Duration of Perfusion Hrs.
Controls:			
1. Normal animal	12	1238 ± 74*	0
2. After anaerobic period and cannulation	7	0 ± 0	0
Perfused with indicated substance after depletion of glycogen:			
3. Sodium beta-hydroxy-butyrate, 100 mg%	6	29 ± 17	4, 6, 12 See Table II
4. Sodium beta-hydroxy-butyrate, 25 mg%	3	5 ± 4	6
5. Glucose, 100 mg%	6	588 ± 81	6
6. Glucose, 100 mg% + Sodium beta-hydroxy-butyrate, 100 mg%	6	595 ± 86	6

* Standard error as calculated from the formula $S.E. = \sqrt{\frac{\Sigma x^2}{N(N-1)}}$, where x equals the difference between the mean and the experimen- value and N is the number of experiments.

atmosphere required for cannulation did not produce any glycogen synthesis from possible products of glycogenolysis contained within the heart muscle.

Line 5 demonstrates that hearts perfused with 100 mgm. glucose per 100 cc. perfusate for six hours synthesized and stored an amount of glycogen equal to 50% of the glycogen content of the heart in an intact untreated animal (line 1). The small amount of glycogen synthesis indicated in the hearts perfused with beta-hydroxybutyrate alone (lines 3 and 4) is of no significance in view of the relatively large standard error obtained. Table I also indicates that whether hearts were perfused with glucose alone or with glucose plus ketone, the same amount of glycogen was formed.

Table II:

This table is an analysis of lines 3 and 4 of Table I. Here it can be seen that 7 of the 9 hearts perfused with ketone showed no glycogen formation, including the one perfused with 100 mgm. per 100 cc. for twelve hours (the values of 14 and 16 mg% are well within the limits of error of the methods used to measure glycogen and glucose). That leaves a significant amount of glycogen in only two of the nine hearts perfused with beta-hydroxybutyric acid as the only metabolite. The concentration of ketone and the duration of perfusion were not significant.

Table III:

This table summarizes the results of studies to determine

TABLE II

An Analysis of Lines 3 and 4 of Table I

Experiment No.	Conc. of Sodium Beta-hydroxybutyrate perfused. Mg%	Duration of Perfusion Hrs.	Ventricle Glycogen Mg%
1	100	4	65
2	100	4	16
3	100	6	0
4	100	6	95
5	100	6	0
6	100	12	0
7	25	6	0
8	25	6	0
9	25	6	14

TABLE III

Effect of Growth Hormone on Glycogen Synthesis from Glucose and Sodium Beta-Hydroxybutyrate in the Perfused Frog Heart

Experiment	Number of Animals	Ventricle Glycogen Mg%	Duration of Perfusion Hrs.
Hearts perfused with indicated substance after depletion of glycogen:			
1. Glucose, 100 mg% and Growth hormone, 2 mg%	3	435 ± 158	6
2. Glucose, 100 mg%, Na beta-hydroxybutyrate, 100 mg% and Growth hormone, 2 mg%	3	548 ± 21	6
3. Na beta-hydroxybutyrate, 100 mg% and Growth hormone, 2+1+1 mg%*	3	166 ± 40	6
4. Na beta-hydroxybutyrate, 100 mg%, Growth hormone, 2+1+1 mg%* and Insulin, 2+2 units**	3	100 ± 44	6
5. Growth hormone, 2+1+1 mg%*	3	139 ± 66	6

* 2 mg% of growth hormone was added at the beginning of the perfusion and 1 mg% at the end of 2 and 4 hours.

** 2 units of insulin were added at the beginning of the perfusion and 2 units at the end of 3 hours.

the effect of growth hormone on glycogen synthesis in the perfused heart. All hearts were first depleted of glycogen by the method previously described. Unfortunately the number of experiments performed in each case was small.

When one considers the large standard error of 158 mg% in line 1 of Table III there is no significant difference between the value of 435 ± 158 mg% of ventricular glycogen obtained with glucose and growth hormone and the 588 ± 81 mg% shown in line 5 of Table I where glucose alone was perfused. Likewise the value obtained when growth hormone was added to ketone and glucose (548 ± 21 mg%, line 2, Table III) is the same as the 595 ± 86 mg% of glycogen synthesized from glucose and beta-hydroxybutyric acid when the hormone was not present (line 6, Table I). Three hearts perfused with growth hormone alone (line 5, Table III) developed an average glycogen content of 139 ± 66 mg%. Combining ketone (line 3, Table III) and ketone plus insulin (line 4, Table III) with the growth hormone did not change the amount of glycogen synthesized.

Table IV:

The results listed in this table demonstrate that frogs injected with 1.0 mgm. of growth hormone intraperitoneally at 0, 24 and 48 hours and sacrificed 8 hours later showed the same amount of cardiac glycogen as suitable controls injected with saline. It should be noted that these control animals (line 1, Table IV) have a much higher level of cardiac glycogen than the animals in line 1, Table I ---2090 ± 65 mg% as compared to 1238 ± 74 mg%

TABLE IV

Effect of Growth Hormone on Cardiac Glycogen of the Frog In-Vivo

Experiment	Number of Animals	Ventricle Glycogen Mg%
Controls:		
1. Fasting frogs injected intraperitoneally with 0.7 cc isotonic saline at 0, 24 and 48 hours and sacrificed 8 hours after the last injection.	3	2090 ± 65
Experimentals:		
2. Fasting frogs injected intraperitoneally with 1.0 mgm. growth hormone per 100 Gms. frog weight (0.7 cc total) at 0, 24 and 48 hours and sacrificed 8 hours after the last injection.	4	2018 ± 144

V. DISCUSSION

A. Depletion of Cardiac Glycogen:

It has long been known that anoxia rapidly lowers the glycogen content of the myocardium (72, 73). In 1937 Bogue, Evans and Gregory (75) demonstrated by means of the dog heart-lung preparation that epinephrine can also deplete the heart of its glycogen. In the present series of experiments advantage was taken of both of these methods to produce glycogenolysis. However, since the epinephrine was simply allowed to drip on the external surface of the frog's heart it is questionable how important a role it played in this respect. Nevertheless, it did appear to strengthen the heart beat which would indicate that it penetrated the muscle cells.

In the desiccator the perfusate of the heart was of course the frog's own blood, the circulation having been left intact. This method of depleting the heart's glycogen worked very well except in a few instances where the flow of blood was sluggish, usually due to hemorrhage in preparing the specimen. In these cases the heart would contract poorly and eventually stop. Such specimens were discarded.

The nature of the white area that developed in the ventricles after the period of anaerobiasis cannot be stated definitely since histological studies were not performed. It resembled an old infarct resulting from coronary occlusion in the mammalian heart in that it appeared ischemic and did not contract. However,

by strict definition an infarct is a local area of necrosis resulting from vascular obstruction. Since the frog has no coronary arteries this was not strictly an infarct, though it resulted from anoxia. Hence I use the word "infarct" in this modified sense and have put it in quotation marks.

In all the hearts analyzed the infarct signaled that the entire ventricular myocardium was depleted of glycogen. In general, the stronger and more rapid the contractions the sooner the infarct developed. However, occasionally a heart was found that did not develop this area of necrosis even after many hours of beating anaerobically, even though it could be demonstrated to be depleted of glycogen. In summary then, all hearts developing infarcts were found to be depleted of glycogen, though the converse was not always true. Thus hearts could continue to beat though free of glycogen. However, when such hearts were perfused aerobically with only the physiological salt solution they beat poorly, often stopping after an hour or two. Therefore no control series of six hour perfusions with just the salt solution could be carried out. In contrast the addition of glucose or ketones to the perfusate caused the hearts to beat strongly for long periods of time.

Clark et al. (70) found that hearts containing glycogen would beat for 24 hours or more with just the physiological salt solution. This would seem to indicate that muscle glycogen is essential to normal cardiac function when no source of energy is present in the perfusate such as ketone bodies, even though the

latter may not give rise to glycogen.

B. Glycogen Levels in the Normal Frog's Heart:

In the work by Clark et al. (76) on the carbohydrate content of the frog's heart it was found that the amount of glycogen varied greatly in different batches of animals and in general was higher in winter than in summer. Also in one of his papers Clark makes the statement that the carbohydrate content rose in proportion to the time the frogs were kept in captivity. However, it is not clear whether the animals were fed or not. In the present perfusion experiments these variations were overcome by first depleting the hearts of all their glycogen.

However, the normal variability of the frog cardiac glycogen probably plays a role in explaining the difference between the 1238 ± 74 mg% of ventricular glycogen in the control animals in Table I and the 2090 ± 65 mg% found in the controls in Table IV. The frogs of line 1, Table I arrived in June and September, 1955 and the glycogen content was essentially the same in both batches. Those in line 1 (and line 2) of Table IV were obtained in September, 1957. Hence frogs in Table I and Table IV cannot be compared. Also it is not known how long the frogs were fasted before they arrived here from Louisiana. A long fasting period would explain the higher cardiac glycogen content of the controls in Table IV. Another difference is that the latter group of frogs received intraperitoneal saline, though Lackey (56) found this did not alter the cardiac glycogen of rats.

The one frog that was fasted for three months (see "Results")

and found to have a much higher concentration of cardiac glycogen than other animals in the same shipment is in line with the frequently made observation that fasting elevates the heart glycogen.

C. Effect of Beta-Hydroxybutyric Acid on Cardiac Glycogen.

Can a net synthesis of glycogen be produced from beta-hydroxybutyric acid? The two hearts that had a glycogen content of 65 and 95 mg% after being perfused with ketone alone provide the only suggestion of such a conversion. When the large amount of glycogen synthesized from glucose (588 ± 81 mg%) is considered plus the fact that 7 of the 9 hearts perfused with ketone alone produced no glycogen, the values of 65 and 95 mg% cannot be construed as evidence that beta-hydroxybutyric acid produces glycogen. Another explanation for this small amount of glycogen must be sought.

One possibility is that the hearts were not entirely depleted of glycogen before they were perfused. Though this cannot definitely be ruled out, it is not likely when one considers that the 7 hearts measured for glycogen content after development of the infarct uniformly showed no glycogen whatsoever. Another explanation is that glycogen was formed from precursors contained within the myocardium. Clark et al. (70) found that glycogen represented only 1/3 of the total reducing substances of the frog heart. Thus, though the infarct indicated the heart was depleted of glycogen other carbohydrates or carbohydrate precursors such as pyruvic or lactic acid may have been present.

It is also possible that the heart formed the glycogen from certain amino acids. However, evidence against such precursors playing a role are the seven hearts in Table II that demonstrated no glycogen synthesis. It is unfortunate that a series of frogs perfused with just the salt solution for a period of a few hours could not be obtained.

Further evidence against synthesis of glycogen from ketone bodies is the finding that growth hormone plus beta-hydroxybutyric acid produced no more glycogen than the hormone alone. Nor was there any evidence of a sparing action of ketone on glucose. This is shown by the fact that a combination of beta-hydroxybutyric acid with glucose produced no more glycogen than glucose alone.

One problem that must be considered is what effect if any the period in the anaerobic chamber had on the normal metabolism of the heart. Were enzyme systems destroyed or adversely affected? Though this cannot be ruled out the excellent synthesis of glycogen from glucose is evidence that the enzyme systems remained intact and functioned normally.

Is it likely that perfusing the heart with acetoacetic acid would have produced results differing from those obtained with beta-hydroxybutyrate? Meyer and Bow (60) found that the dog's heart metabolized acetoacetic acid by degradation to 2 acetyl-CoA which then entered the Kreb's cycle. Waters et al. (48) and Barnes et al. (49) found that beta-hydroxybutyric acid was readily oxidized by the dog heart. The present series of experiments indicate the same is true for the frog's heart since hearts

perfused with ketone contract much more strongly and for a longer time than hearts perfused with salt solution alone. It has also been noted (61) that butyrate is only metabolized by animal tissues by beta-oxidation to 2 acetyl CoA. By combining these facts it becomes evident that beta-hydroxybutyric acid must be oxidized to acetoacetic acid and not reduced to butyric acid in order to be utilized by the heart. Since the heart therefore converts beta-hydroxybutyrate to acetoacetate it is highly unlikely that perfusion with the latter substance would have given results differing from those obtained with the ketone body that was used. In this connection Lackey (56) found essentially the same increase in cardiac glycogen when either beta-hydroxybutyric or acetoacetic acid was infused.

Would acetone have given rise to glycogen by formation of pyruvate? Meyer and Bow (58, 60) found no evidence suggestive of a conversion of acetoacetate to acetone to pyruvate even though, as was previously mentioned, acetoacetic acid is decarboxylated to acetone by animal tissues to a small extent. Hence it is unlikely that acetone would have formed glycogen in these experiments when beta-hydroxybutyric acid did not.

D. Growth Hormone:

The experiments with growth hormone (Tables III, IV) do not confirm the findings of Russell and coworkers in rats that this substance increases the level of cardiac glycogen by acting as a hormone. As has been pointed out under "Results" the addition of growth hormone to glucose or

to glucose plus ketone did not increase the amount of glycogen synthesized. Though the hormone plus ketone produced a significant amount of glycogen whereas ketone alone did not, the amount formed was the same as was obtained with the hormone alone. Hence no evidence was found indicating that growth hormone increases glycogen synthesis from dextrose or stimulates formation from ketones. The in-vivo experiments (Table IV) also gave no evidence of increased cardiac glycogen in animals injected with growth hormone. Insulin also was inactive.

Line 5 of Table III suggests a synthesis of glycogen from the amino acids of growth hormone, a finding reinforced by lines 3 and 4 of the same table. Assuming the ventricle weighed 0.5 Gm. (rarely did it weigh slightly more), a glycogen level of 166 mg% (line 3, Table IV) would mean a total glycogen content of 0.83 mgm. Four milligrams of growth hormone per 100 cc. of salt solution was perfused during the 6 hour period or a total of 16 mgm. of hormone since 400 cc. of perfusate was used in each experiment. This is a more than sufficient amount to explain the synthesis of glycogen even if it is assumed the atria synthesized a similar amount. That growth hormone in combination with glucose did not produce more carbohydrate than glucose alone might be interpreted to mean that the heart will only synthesize a fixed amount of glycogen in a limited amount of time no matter how many precursors are available.

Why did these experiments fail to confirm Russell's finding that growth hormone produces an increased level of cardiac glycogen? First there is the problem of species difference.

Russell worked with rats; these experiments were done with frogs. The failure of the in-vitro experiments to confirm Russell's findings might be attributed to growth hormone stimulating glycogenesis from some substance not present in the perfusion system. However, this will not explain the failure to demonstrate elevated cardiac glycogen levels in the frogs injected intraperitoneally with the hormone. In connection with the in-vivo experiments it should be noted that the control animals in line 1 of Table IV have a very high glycogen content. Perhaps hearts with a lower concentration of carbohydrate would have synthesized or stored glycogen more readily in response to growth hormone. In both the in-vivo and the in-vitro experiments the dosage of hormone was comparable to that used by Russell et al.

Russell and Bloom (68) concluded that growth hormone probably increased cardiac glycogen by blocking its utilization. It may be that under the conditions of the perfusion experiments the heart would not readily utilize the glycogen it had synthesized and therefore growth hormone would be unable to play a role. It would be interesting to see if growth hormone interfered with the process of deglycogenation in the anaerobic chamber. Also it may be that the preparation of hormone used was inactive in frogs.

VI. CONCLUSIONS

As regards amphibian cardiac function the following conclusions may be drawn:

1) An anaerobic atmosphere plus epinephrine rapidly depletes the heart of its glycogen. The development of the infarct serves as an excellent indicator that the ventricle is devoid of this carbohydrate.

2) Even when entirely depleted of glycogen the ventricle need not resynthesize it in order to contract. Its function, however, is greatly improved by the addition of a substance such as glucose or beta-hydroxybutyrate to the perfusing fluid. Hence, the amphibian heart appears able to utilize ketone bodies for energy as can the mammalian heart.

In reference to the question of a net synthesis of glycogen from ketone bodies it is concluded that under the conditions of the present series of experiments no evidence for such a conversion could be obtained, even though the myocardium first was depleted of all glycogen. Therefore these experiments add to the generally held opinion that the animal body is unable to bring about such a synthesis. Nor was any sparing effect of ketone on glucose found.

It is also concluded that under the present experimental conditions growth hormone, in its role as a hormone, could not be demonstrated to elevate the cardiac glycogen in the frog. This is in contrast to the finding in the mammal (67, 68, 69) where growth hormone does increase the store of glycogen in the

heart, the increase being due to its hormonal action. However, the frog's heart may have synthesized glycogen from the growth hormone itself.

The problem of why animals in ketosis show an increase in cardiac glycogen remains unsolved though the work by Russell et al. sheds much light on the problem. Certainly the increase does not appear to be due to a direct conversion of ketone bodies to carbohydrate.

VII. SUMMARY

The hearts of American bullfrogs were first depleted of their glycogen content by placing the animal in an anaerobic environment and permitting epinephrine to drip on the exposed heart. It was found that the development of an "infarct" in the ventricle served to indicate when the myocardium was devoid of glycogen. The heart was then removed from the animal and perfused with glucose, beta-hydroxybutyric acid and a combination of the two. From glucose and from glucose plus ketone the heart ventricle in six hours synthesized an amount of glycogen equal to 50% of that found in the normal frog ventricle. However, no synthesis could be demonstrated from ketone alone.

The addition of growth hormone to the perfusate did not increase the synthesis of glycogen from glucose or induce any formation from ketone. Nor did the intraperitoneal injection of the hormone into fasting rats result in increased levels of glycogen in the myocardium.

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